Central Nervous System Injury:

An in vivo and ex vivo analysis of axon regeneration

Undergraduate Honors Research Thesis

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By

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Abstract:

Extensive damage to a developed spinal cord can be devastating. The axons of nerve cells cannot regenerate effectively following injury, leading to impaired sensation and/or movement. More research is needed on the mechanisms underlying injury, and promising therapeutics are still lacking. Many molecules have been implicated in the inhibition of growth at the injury site. One such class of molecules are Chondroitin Sulfate Proteoglycans (CSPG). Interestingly, another group, Heparin Sulfate Proteoglycans (HSPG), has been shown to be growth *promoters* that act on the same receptor, Protein Tyrosine Phosphatase Sigma (PTPSigma).

Given this literature, it is seen that PTPSigma acts in a bimodal manner by binding both a growth inhibitor (CSPG) and growth promoter (HSPG). The purpose of the first part of the project was to test whether the injection of additional HSPG, named Glypican-2, at the injury site of a rat spinal cord will alter the signaling of PTPSigma to favor axon growth and counteract the inhibition of CSPG. We found that Glypican-2 promoted axon growth for tyrosine hydroxylase (TH) and serotonin (5HT) fibers while mitigating the dieback of corticospinal tract (CST) fibers. Furthermore, tau and Alzheimer's Precursor Protein (APP) have been linked to growth inhibition of neurons following injury. When compared to levels of CST dieback, the decrease in tau and APP levels with treatment show a significant connection. This suggests that GPC-2 may be mitigating the normally negative effects that these proteins have post-injury on neuron growth. These data support Glypican-2 as a promising therapeutic for spinal cord injury.

Furthermore, current models often utilize a dorsal root ganglion (DRG) culture or an embryonic cortical neuronal assay to assess axon growth. Because the growth potential of adult neurons are much lower than the embryonic, and it is much more difficult for CNS neurons to survive and grow their axons, it is imperative to formulate an ex vivo assay for adult CNS

neurons. The second part of the project consisted of developing an ex-vivo central nervous system (CNS) culture with electrical stimulation to further examine the effects of inhibitors such as CSPG in a novel model *outside* of the organism. The progression of this project was halted by limited solidification of collagen gel, which was needed to suspend the nervous tissue. While substantial data was not collected at this stage, the study was still valuable in providing insight into the potential issues that may result in ex vivo study and opportunities for modifications.

Introduction:

Over 1,000,000 Americans suffer from spinal cord injury, and it is responsible for 27% of the cases of paralysis in the United States (www.christopherreeve.org). Currently, the damage is essentially irreversible because affected neurons are not able to grow back into their original positions and recover function (Silver and Miller 2004). Much research has gone into the inhibitory factors preventing regeneration in the brain and spinal cord compared to the peripheral nervous system, where regeneration occurs naturally (Huebner and Strittmatter 2009).

Many inhibitory components have been analyzed for their role in axon growth. One such group is Myelin-Associated Inhibitors (MAIs), which are present in the myelin of the Central Nervous System and play a role in axon sprouting (Geoffroy and Zheng 2014). The three specific inhibitors studied are Nogo, MAG, and OMgp. Knockout studies of these animals have shown varying levels of success in recovery. Their deletion is more strongly tied to axon sprouting, which is growth from another point on the damaged neuron or from another neuron, rather than regeneration, growth from the point of injury (Geoffroy and Zheng 2014).

Another inhibitory component analyzed has been Chondroitin Sulfate Proteoglycan, which is a large, negatively charged molecule (Sharma 2012). The presence of CSPGs vary on a temporal and situational context. In the developing nervous system, their expression is initially heightened as they guide axons along the proper trajectory through repulsion but then decreases with maturation. After injury, CSPGs are increasingly produced and have been shown to inhibit axon regeneration (Shen 2014). The counter molecule to CSPGs are Heparin Sulfate Proteoglycans (HSPGs), which attracts axons during development and have been shown to possess growth promoting characteristics (Shen 2014).

These two molecules function in opposite, but necessary ways during neural development that are essential to the proper construction of the nervous system. However, after injury, these opposite effects do not work in conjunction. These attributes led to studies examining what receptor binds the molecules. It was found that Protein Tyrosine Phosphatase Sigma (PTPSigma) binds *both* CSPGs and HSPGs (Shen et al 2009, Shen 2014). This was a significant discovery because not only did it provide a specific therapeutic target for spinal cord injury, but demonstrated the ability of the receptor to act in growth promotion or inhibition depending on the amount of activation.

Past research has shown that the two compounds compete to control the oligomerization of the PTPSigma receptor (Shen 2014, Coles & Shen et al 2011). The treatment of the brain post-ischemia with Glypican, an HSPG, had positive effects on growth, regeneration, and function (Hill et. al 2012). This therapeutic strategy has not been tested extensively in spinal cord injury models. The first part of the project aimed to examine whether the additional supply of growth promoting HSPG, Glypican-2, leads to regeneration following spinal cord injury and to analyze molecular factors impacting this process.

Furthermore, while in vivo models are often utilized for studying injury, in vitro models are lacking. Current models examining central nervous system injury often utilize a dorsal root ganglion culture (DRG) to assess axon growth (Melli 2009) or an embryonic cortical neuronal assay (Sciarretta and Minichiello 2010). However, the mean age affected by spinal cord injury, for example, is forty years old (www.brainandspinalcord.org). Therefore an adult animal would simulate the wider-afflicted clinical population in humans. Past adult models have not shown sustained survival in many cells past seven days (Sypeca et al 2015, Brinn et al 2016). As a result, a new approach is needed to promote growth. Previous research has shown that electrical

stimulation is a promising avenue for neural tissue regeneration (Sirivisoot et al 2014). Peripherally, stimulation has led to regeneration (Chen 2011, Willand et al 2016). Thus, we proposed a setup in which constant electrical stimulation will be applied to the cortical tissue to test whether this strategy promotes cell growth.

Method:

Experimental Setup/Treatment Delivery: (Note: Injury and drug delivery procedure was carried out by Dr. Philip Popovich's lab, Department of Neuroscience, Ohio State). Thirteen adult female Sprague Dawley rats were hemi-lesioned at the 8th thoracic vertebrate level on the left side (LHx). The injury was done as a hemitransection so that the right half of the cord could serve as a control. Rats were randomly selected to be given type 1 rat tail collagen treatment or GPC-2 mixed in collagen. Each rat was given ~2 µl of 2 µg/µl collagen (with or without 0.5µg/µl GPC-2) to fill the injury gap, which solidified within 2 min at 37.5°C. Then, 4 more µl was used to seal the area surrounding the injury gap. The treatment group received 3 ug of GPC-2. BBB motor scores were measured at week 1, 2, 5, and 6 after injury. 4 weeks after injury, Biotin Dextran Amine (BDA) (2ul, 10%,10k MW) was injected into the right (contralateral) sensorimotor cortex region to mark descending motor axons of the corticospinal (CST) tract. 8 weeks post-injury, rats were perfused. 15mm of spinal cord tissues with lesion site at the center were taken for horizontal sectioning at 10µm thickness. The setup is summarized in Table 1 and Figure 1 below.

Experimental Group	T8 LHx Collagen alone	T8 LHx Glypican-2 in Collagen	Total
Number of rats (N)	6	7	13

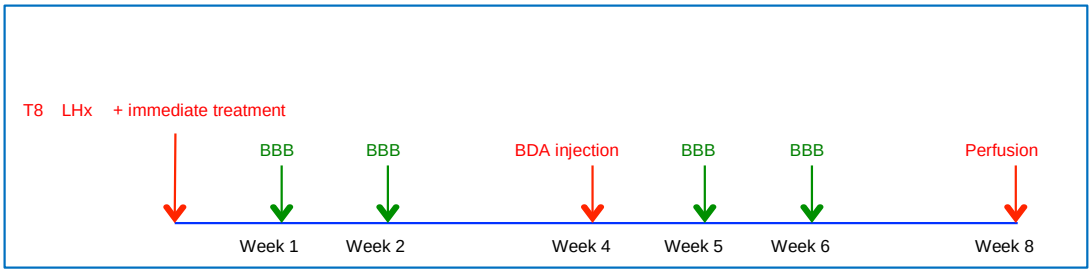


Table 1: Overview of Experimental Groups; **Figure 1:** Outline of Experiment Timeline

Immunohistochemistry Analysis: The following general staining protocol was carried out for all the labeled fibers and proteins. First, slides were warmed on the slide warmer for 30-60 min at 37 degrees Celsius. The sections were each bordered with Pap Pen to ensure maximum coverage of antibody. Then, the slides were rinsed with .02 % triton X100 in PBS (10 min x 2). The slides were incubated in blocking buffer (5% NDS, 5% NGS, .2% TritonX100 in PBS) for 1 hour at room temperature. Primary antibody was diluted in blocking buffer, aliquoted in EPP tubes, and spun in the cold room at 15K speed for 5 minutes. The samples were incubated with this solution overnight at 4 degrees Celsius. The next day, slides were washed with .02 % tritonX100 in PBS (10 min X 4-5). Secondary and tertiary antibody was diluted in blocking buffer, aliquoted in EPP tubes, and spun in the cold room at 15K speed for 5 minutes. The slides were incubated with this solution for 2 hours at room temperature without exposure to light. Then, slides were washed

with excessive amounts of PBS (10 min x 4-5). Mounting solution was placed on the sections, followed by the coverslip. The slides were stored at 4 degrees Celsius until imaging.

First, tyrosine hydroxylase (TH) and serotonin (5HT) axons were labeled via immunofluorescence to evaluate their growth proximal and distal to the injury site. CST motor fibers were also marked via the labeling of BDA. In addition, tau protein was also analyzed. Tau protein has been implicated as a marker of brain injury (Shiiya et al 2004). Elevated tau levels have been positively correlated with the damage in spinal cord injury (Roerig et al 2013). Thus, it is imperative to test tau to explore whether the Glypican-2 treatment has an effect on its levels, which may reflect the extent of injury. Last, Alzheimer's Precursor Protein (APP) was also tested. High levels of APP post-injury in the spinal cord has been linked to decreased functional recovery when its breakdown is inhibited (Pajoohesh-Ganji 2014). Furthermore, a study showed a heightened presence of beta APP in axons after spinal cord injury and may suggest that the normal molecular transport down these axons is negatively impacted, leading to dysfunction (Ahlgren et al 1996). Thus, APP levels may also convey additional information about the impact of Glypican-2 on the extent of injury and molecular factors within the central nervous system after injury.

Imaging: The fluorescently-labeled sections were imaged via Zeiss Zen Software on a Zeiss Confocal Microscrope at 10X. The TH and 5HT axons were imaged at 10X. The Tau and APP signals were also imaged at 10X and scans of the proteins were taken based on the area of highest intensity signal via pixel values.

Quantification/Analysis: The number of segments for Tyrosine Hydroxylase and Serotonin fibers were counted, and their distances from the lesion site were measured via the Zeiss Zen Software

measuring tool. The Tau and APP imaged areas were quantified for average intensity (pixels/ area) using Image J software. The obtained data from TH, 5HT, and both proteins were analyzed with two-tailed Student's t-test and standard error calculations. Significant effects were reported using $p < 0.05$.

Part 2: Ex-vivo Assay

Motor Cortex extraction: The mice were euthanized via CO_2 exposure, followed by cervical dislocation. Animals were sprayed with 70% EtOH, then decapitated. An incision was made along the midline towards the rostral end of the head. Then, scissors were then used to cut through the skull. Forceps were used to detach the brain from the surrounding gray matter and remove it. The brain was placed in a dish with cool PBS on ice. A scalpel was used to first cut off the olfactory bulb, then make a coronal section to isolate the motor cortex, utilizing a scalable brain atlas online (https://scalablebrainatlas.incf.org). The motor cortex was isolated because the CST neurons proved to be the most difficult to regenerate in the first part of the study. Thus, a cortical motor neuron assay was imperative.

Collagen Gel Preparation: Deionized water was autoclaved. To make the collagen gel solution, collagen gel (3.83 mg/mL), Accel Medium, and $NaHCO_3$ was added in an 8:1:1 ratio. This step was refined to obtain the optimal collagen gel concentration and pH. The solution was kept on ice until it was added to the well plate.

Experiment Setup: An electrode was placed at the bottom of a well in the 24 well cell culture plate. 500 μL of gel mix was pipetted in the well. Then, the isolated motor cortex tissue was put on top. The second electrode was placed on top of the motor tissue in the solidifying collagen gel solution. Last, 1000 μL of Accel medium was added. The plate was taken to the incubator and stored at 37

degrees Celsius with 5% CO_2. The incubation time was varied to determine the optimal period for gel solidification.

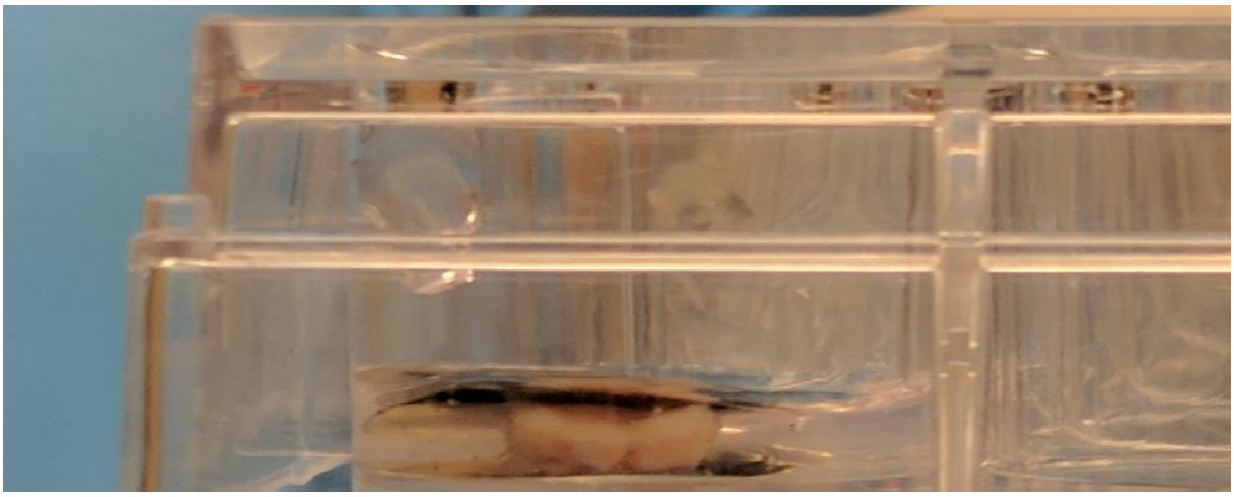


Figure 3: Visualization of the experiment setup. The motor cortex piece is sandwiched between two electrodes while being suspended in a collagen gel solution. While not pictured above, Accel medium was added above the top electrode.

Contamination: The plates were checked daily for contamination by noting a change in the color of the solution from the normal pink appearance.

Electrical Stimulation: While this step was unfortunately not reached, the plan was to continue in the following way. The tissue will be stimulated at a constant frequency of 40 Hz for a variable amount of days to assess impact on cell growth. A past study showed that healthy tissue produces circuit oscillations at 40 Hz, and this normal process is disrupted in injury (Iaccarino et al 2016).

Immunohistochemistry/Analysis: The plan was to proceed with the following analysis. Inhibitors to regeneration post-injury such as CSPG will be introduced to the section and their effects on neuron growth will be assessed. The method of tissue clearing will be first utilized as a data collection strategy. If this is not successful, the traditional sectioning, staining, and imaging

processes listed above will be followed to determine the effect of inhibitors on a novel ex-vivo adult CNS assay.

Results:

5HT/TH: There are increased number of axon fibers in the lesion core for the rats treated with Glypican-2 and collagen, compared to just collagen alone (Figure 2A, student's t test p=.06; Figure 3A and 3B). Given the location and shape of the fibers in the lesion core, we believe these are regenerating axons. The fibers are also present in the area below the lesion core (Figure 3C and 3D). Here neurites are believed to be either regenerating or sprouting from the opposite side. The increase in specifically TH fibers in this region is significantly greater in the treatment compared to non-treated (Figure 2B; $p < .05$; Figure 3C and 3D). A major obstacle to growth is the inability of axons to cross the lesion site (Silver and Miller 2004). These results suggest a growth promoting role of GPC-2 on serotonin and tyrosine hydroxylase axons around and into the injury site.

CST: While CST fibers did not penetrate the lesion site, they experienced less dieback or retraction from the injury site than the collagen only group (Figure 4C; $p > .05$). Immunohistochemistry shows that more axons approached the inhibitory lesion site compared to the controls and populated the boundary (Figure 4A and 4B). This suggests that GPC-2 is promoting CST growth closer to the injury site and may play an important role in stimulating regeneration of CST axons by providing a favorable environment for development after injury.

Figure 2. GPC-2 stimulates 5HT fiber regeneration.

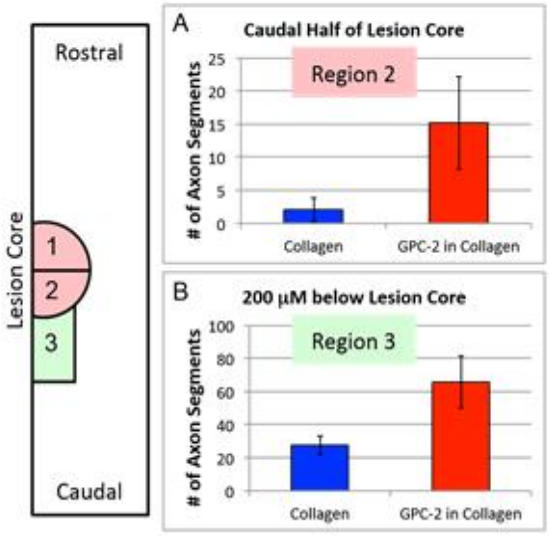


Figure 3. GPC-2 stimulates TH fiber regeneration.

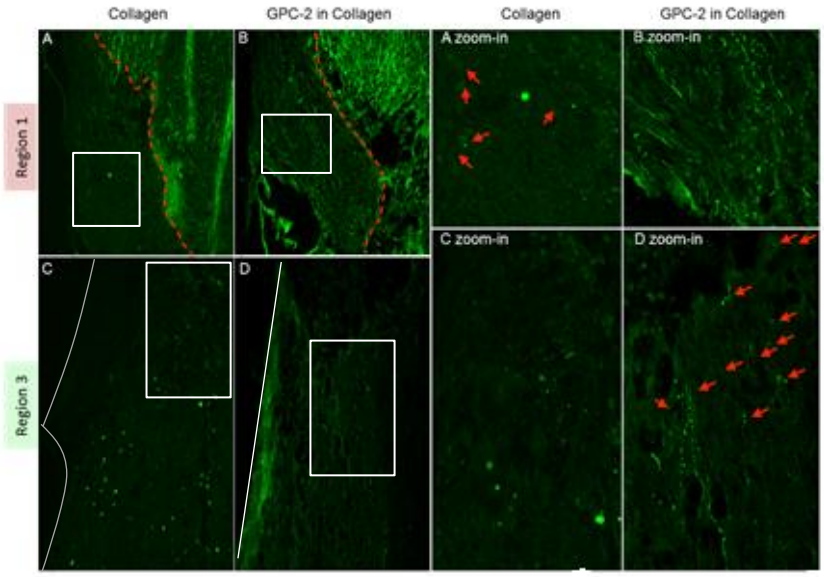
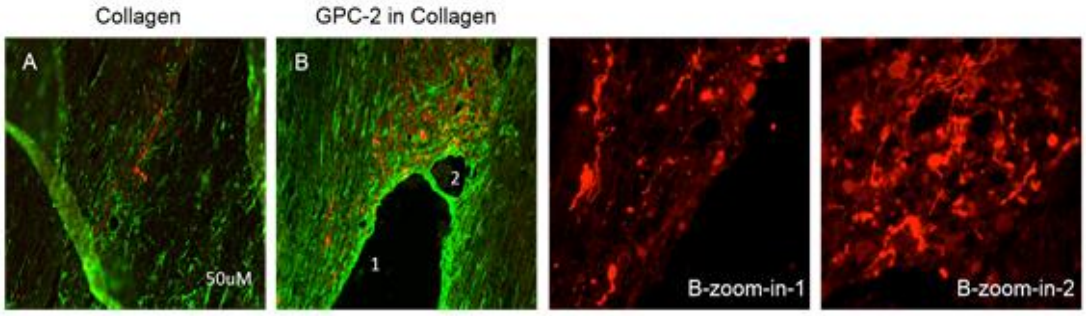


Figure 4. GPC-2 reduces dieback of CST fibers.



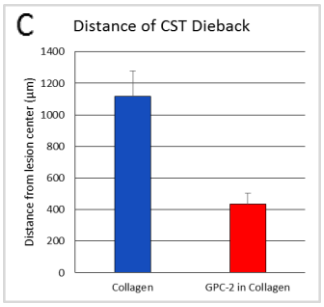
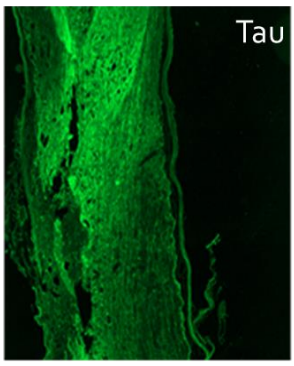


Figure 2: Diagram of horizontal section shows regions analyzed for 5HT and TH axons. Fibroblast-filled GFAP-negative lesion core is shown in pink. Rostral half: region 1; caudal half: region 2. Region 3 (green) is GFAP-positive area immediately below region 2 that extends 200µM caudally and is left to midline/central canal. For each rat, 7 sections spacing 100µM in between from the central 700µM of the cord (850µM inward from dorsal and ventral surface) were counted for numbers of axon segments. Numbers of 5HT axon segments (sum of all sections) in region 2 and 3 are shown in Fig.2A and B respectively. **Figure 3:** Stains of TH axons from region 1 and 3. Solid white lines delineate edge of cord sections. Boxed areas are shown in larger magnification to the right. In Fig.3, red dotted lines mark the border between GFAP-positive reactive astrocyte area (upper-right) and fibroblast-filled lesion core (lower left). White dotted lines in zoomed images delineate border of lesion gaps. Red arrows point to axons, axons obviously visible are not pointed. For zoom-in of Fig.3B and 4B, no arrows are used due to too many axon segments**. Figure 4:** CST axons shown approaching fluid-filled lesion gap (stained red. For Fig.4C, for each rat, distance from the closest BDA axon(s) to lesion margin was measured by Zeiss Zen software. Error bars: standard error. Student t test *p* value: Fig.2A (region 2), 0.06; Fig.2B (region 3), 0.03; Fig.4C, 0.13. Collagen group, n=6; GPC-2 group, n=7.

Tau: The mean of the maximum tau protein levels for each rat decreased between the collagen only group and the GPC-2 with collagen group but not significantly (Figure 7; student t test, $p > .05$). Stained tissue images are shown in Figure 5, illustrating the qualitative decrease in protein level. However, when the CST dieback distance is compared to tau levels, the relative ratio is significantly lower for the treatment group compared to the collagen only group, suggesting that GPC-2 may be playing a role in decreasing the detrimental impact of tau on CST dieback (Figure 9; $p = .007$).

APP: Similar to tau, the mean of the highest APP levels for each rat decreased between the collagen only group and the GPC-2 with collagen group, but the difference was not significant (Figure 8; $p > .05$). Stained tissue images for APP are shown in Figure 6 and demonstrate the decrease qualitatively. However, just as in the case of tau, when the CST dieback is compared to APP levels, the resulting ratio is significantly decreased in the treatment group compared to the collagen only group, suggesting the role of the treatment in mitigating APP's negative impact on CST retraction (Figure 10; $p = .019$).



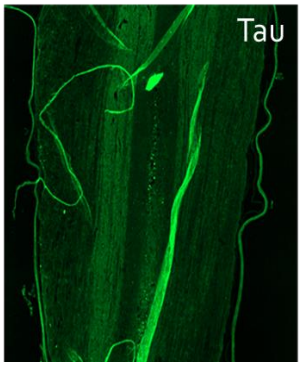
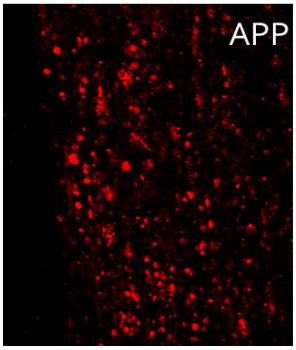


Figure 5: Tau levels with collagen only (left) and GPC-2 with collagen (right)



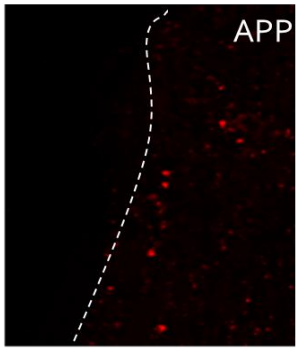


Figure 6: APP levels with collagen only (left) and GPC-2 with collagen (right)

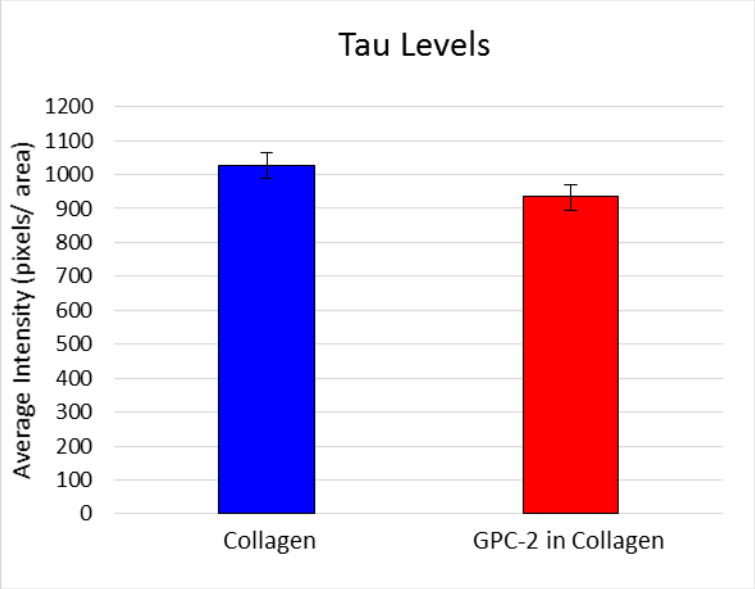


Figure 7: Decrease in Tau levels with treatment. Tau Quantification by mean intensity (pixels/ area)

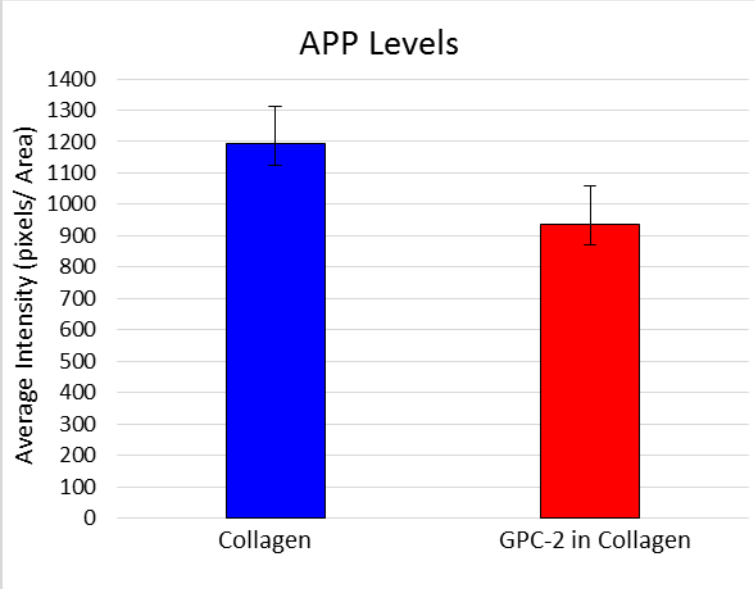


Figure 8: Decrease in APP levels with treatment. APP Quantification by mean intensity (pixels/ area)

Figure 9: GPC-2 Decreases Tau's effect on CST Dieback

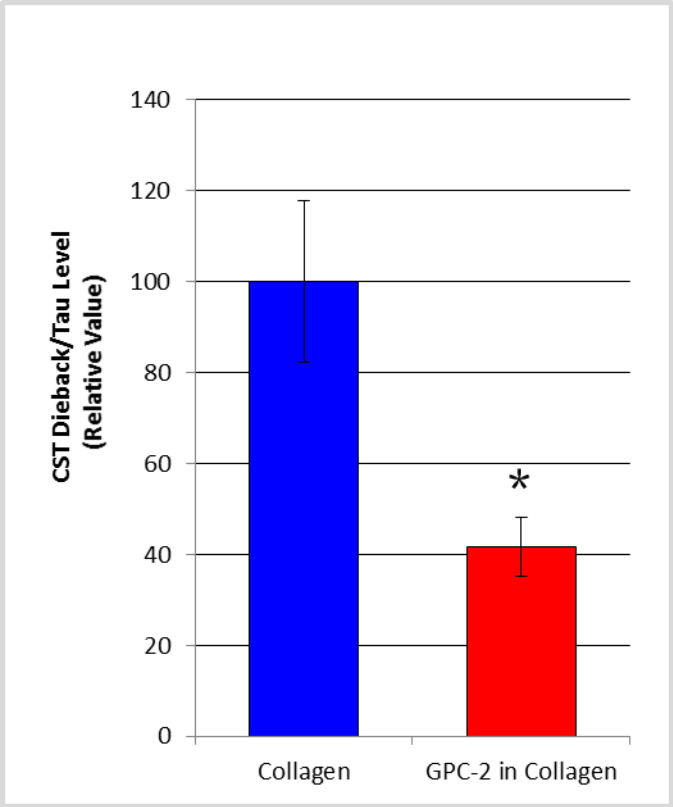


Figure 10: GPC-2 Decreases APP's effect on CST Dieback

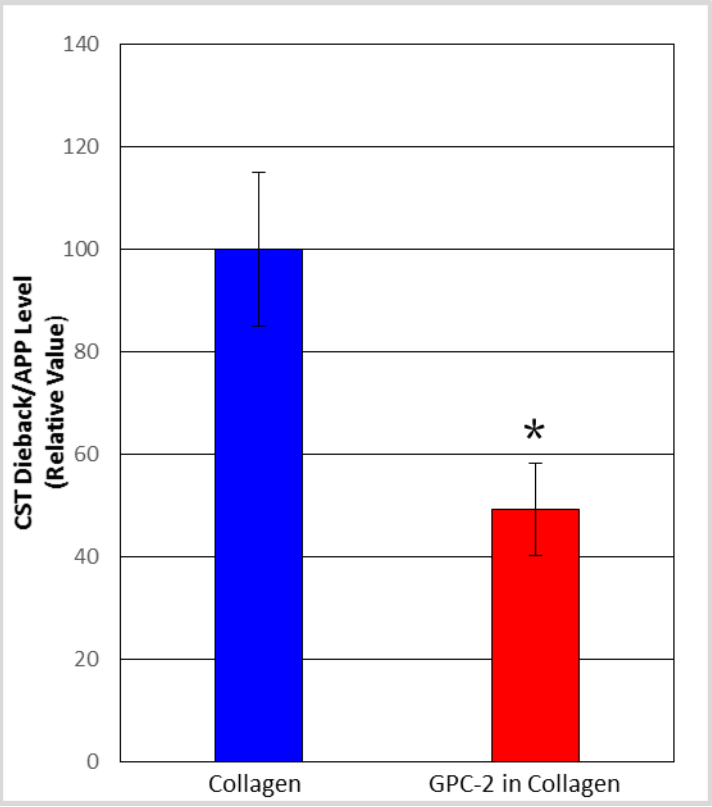


Figure 9 and Figure 10: CST dieback/protein level scaled to 100 to compare relative impact. Error bars: standard error. Student t test *p* value (p=.07, p=.019, respectively); Collagen group, n=6; GPC-2 group, n=7.

Ex vivo assay: The tissue culture combined with electrical stimulation was unable to be completed because the collagen gel did not solidify in order to suspend the tissue. Multiple efforts were made to adjust the parameters to promote gel solidification (Table 2). The pH of the $NaCO_3$ was confirmed to be between 8 and 9. The collagen gel was tested to be at a neutral pH of 7. The gel was tested at a density of 1 mg/mL and 2 mg/mL, but neither was able to produce success. The incubation period was also varied. Among all attempts, contamination was not noted.

Table 2: Ex Vivo Assay Results

Variable Tested	**Manipulation/Test**	**Solidification**
pH	Checked $NaHCO_3$ pH (8-9) Adjusted and verified collagen gel solution pH to be neutral	No
Incubation Period	Varied from 15 min to 1.5 hours; left overnight	No
Density	Tested at both 1 mg/mL and 2 mg/mL	No

Discussion:

The purpose of the study was to test the therapeutic potential of supplying an HSPG, Glypican-2, to the injury site immediately following spinal cord injury. Past studies have established the significant inhibitory properties of Chondroitin Proteoglycans (CSPGs) following spinal cord injury (Siebert et al 2014, García-Alías 2012, Bradbury et al 2002). Efforts to downregulate CSPGs after injury have led to moderate functional recovery and axon sprouting and regeneration (Bradbury et al 2002, Barritt et al 2006, Tom et al 2009). The finding of PTP Sigma as a receptor for CSPG provided a direct receptor signaling site for the molecule (Shen et al 2009). HSPGs have growth-promoting properties and have been shown to act competitively with CSPGs over the oligomerization of the PTPSigma receptor (Shen 2014, Coles & Shen et al 2011). Studies in a rat stroke model showed that treatment with the HSPG Glypican promoted improvements anatomically, histologically, and functionally (Hill et al 2012). However, a rat model of spinal cord injury has not been extensively studied with this therapeutic approach.

The results underscore the potential of this treatment strategy while also illuminating the factors that proteins such as tau and APP may have in the process. Given that the conceptual foundation of this study is based on the bimodal nature of the receptor PTP Sigma, these results are particularly promising (Shen 2014). It points to a specific receptor that can be targeted for effective therapy.

The growth into and past the lesion seen among TH and 5HT fibers is noteworthy. While the GPC-2 treatment did not promote CST growth into the lesion site, there was less retraction away from the injury. These results suggest that GPC-2 is effective in promoting growth among the different groups of fibers. Furthermore, when CST dieback levels are compared to tau and APP levels, the differences between the treatment and non-treatment group are significant. There is less dieback among CST fibers per unit tau and APP in the HSPG group. This suggests that GPC-2 may be mitigating the normally negative effects that these proteins have on post-injury neuron growth as previous studies have shown (Shiiya et al 2004, Roerig et al 2013, Pajoohesh-Ganji 2014, Ahlgren et al 1996).

The second part of the experiment, the ex vivo assay, aimed to construct and refine an in vitro growth condition that supported the survival of adult neurons. This is needed given that current research utilizes mainly embryonic (Melli 2009) or dorsal root ganglion cultures (Sciaretta and Minichiello 2010). Efforts with adult cultures have had limited success (Sypeca et al 2015, Brinn et al 2016). Previously seen in peripheral nerve research, utilizing electrical stimulation to promote CNS growth was a novel addition to this study (Chen 2011, Willand et al 2016). This process unfortunately did not succeed due to the limited solidification of collagen gel. While this problem is prevalent, the suggested troubleshooting mechanisms did not improve the result

(Artsym and Matsumoto 2010; Farhat 2011: www.protocol-place.com). The pH, incubation time, and density of the gel were all varied, but solidification was not observed. Given the use of DMEM in multiple collagen gel protocols, it is possible that Accel medium is not well-suited for this process (Artsym and Matsumoto 2010; Farhat 2011: www.protocol-place.com,). A note of success is that the absence of contamination does suggest effective sterilization technique and rules out major microbes as reasons for the lack of gel solidification.

Potential limitations of the study may include the number of tested rats. Six control and seven treatment rats were utilized. While not a large sample size, the study was completed to explore the potential therapeutic effect of Glypican-2. Given these results, future studies can be expanded. Furthermore, CST regeneration was limited and did not penetrate the lesion. However, the decreased retraction away from the lesion was seen as promising. In the second portion of the experiment, the progress was limited by the technical step of gel solidification.

Future directions include manipulating the dose of GPC-2 to test effect on post-injury axon regeneration. Furthermore, the mechanisms by which tau and APP impact neurite growth can also be explored. Last, the ex vivo assay's protocol can be refined in the future in order to ensure gel solidification and the continuation of the proposed experiment.

These results contribute to research on a condition that currently affects over 1,000,000 Amercians every year (www.christopherreeve.org). When taking into account traumatic brain injury and stroke, over 2,500,000 cases of injuries to the central nervous system occur each year (www.mbi.ufl.edu/). In severe cases, the disabilities cannot be overcome due to the limited regeneration of axons (Silver and Miller 2004). Thus, given the prevalence of these conditions and the paucity of effective treatments, research such as this project into central nervous system

regeneration is imperative. It provides a treatment strategy and therapeutic for combating the injury in a safe, logical manner. Information learned from this study regarding the various molecular factors in the body, whether growth promoting or inhibiting, can be applied to our general understanding of the growth and development of the nervous system and be used as a tool for research into other conditions that disrupt its normal functioning. In this way, the project has implications for not only injury studies, but for the broader field of neuroscience in treating neurological conditions.

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